United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 201

Supplemental Assay Method for Potency Testing *Clostridium perfringens* Type C Beta Antigen

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Supplemental Assay Method for Potency Testing *Clostridium perfringens* Type C Beta Antigen

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1. Introduction

Supplemental Assay Method for Potency Testing *Clostridium perfringens* Type C Beta Antigen

This Supplemental Assay Method (SAM) describes the method used to determine whether biological products containing *Clostridium perfringens* type C beta antigen can stimulate the production of satisfactory immunity as prescribed by the Code of Federal Regulations, Title 9 (9 CFR), Part 113.111. For products that require 2 vaccinations, rabbits are vaccinated twice 20-23 days apart and bled 14-17 days following the second vaccination. For products that require a single vaccination, rabbits are vaccinated and bled 34-40 days later. The serum is titrated by a toxin-antitoxin neutralization test, using mice as an indicator.

2. Materials

2.1 Equipment

2.1.1 Mixer, vortex-type

2.1.2 Centrifuge

2.1.3 Autoclave

2.1.4 Freezers, -20°C and -70°C

2.1.5 Refrigerator, 2°-7°C

2.1.6 Micropipettes, 100-µl and 1000-µl

2.2 Reagents/supplies

2.2.1 *C. perfringens* type C standard beta antitoxin, IRP 486

2.2.2 *C. perfringens* type C standard beta toxin, IRP 513(04)

2.2.3 Peptone diluent

2.2.4 Screw-top Erlenmeyer flask, 500-ml, with cap

2.2.5 Syringes, needle-locking, 1-cc, 10-cc, 20-cc, or 30-cc

2.2.6 Needles, 25- to 27-gauge x 1- to 1 1/4-inch, 20-gauge x 1 inch

2.2.7 Vacutainer® needles, 20-gauge x 1 1/2-inch

2.2.8 Serum separation tubes, 12.5-ml

2.2.9 Pipettes, 5-ml, 10-ml, 25-ml

2.2.10 Tips for micropipettes

2.2.11 Ketamine hydrochloride, 100 mg/ml solution

2.2.12 Xylazine, 20 mg/ml solution

2.2.13 Water, distilled or deionized, or water of equivalent purity

2.2.14 Polystyrene snap-top tubes, 17 x 100-mm with caps

2.2.15 Polystyrene snap-top tubes, 13 x 100-mm with caps

2.2.16 Polystyrene screw-cap conical tubes, 17 x 120-mm

2.3 Test animals

2.3.1 New Zealand White rabbits, nonpregnant females, 4-8 lb (Eight rabbits are required per serial to be tested.)

2.3.2 White Swiss nonpregnant female mice, 16-20 g (Five mice are required for each toxin-antitoxin mixture.)

3. Preparation for the test

3.1 Personnel qualifications/training

Technical personnel need a working knowledge of the use of general laboratory chemicals, equipment, and glassware and must have specific training and experience in the safe handling of clostridial toxins. Personnel must have specific training in the care and handling of laboratory rabbits and mice.

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3.2 Preparation of equipment and supplies

3.2.1 Sterilize all glassware before use.

3.2.2 Use only sterile supplies (pipettes, syringes, needles, etc.)

3.2.3 Operate all equipment according to the manufacturers' instructions.

3.3 Preparation of reagents

3.3.1 Peptone diluent

Peptone (Difco)	8 g
NaCl, reagent grade	2 g
Water q.s. to	800 ml

Dissolve peptone and sodium chloride in water. Adjust pH to 7.2 with 1N sodium hydroxide. Fill 500-ml Erlenmeyer flask no more than 3/4 full with diluent. Autoclave with caps loosened at 121°C for 25-30 minutes. Cool flasks and tighten caps. Store at 2°-7°C for up to 3 months.

3.3.2 Preparation of *C. perfringens* type C standard beta antitoxin

1. *Clostridium perfringens* type C beta antitoxin IRP 486 contains 500 units of beta antitoxin per ml (AU/ml) and has been standardized against the World Health Organization *C. perfringens* (*C. welchii*) type C International antitoxin. Each vial contains 1.3 ml of antitoxin.

2. A dilution of standard beta antitoxin containing 10 (AU/ml) is used in the toxin-neutralization test. To prepare the initial dilution, add 1.0 ml of well mixed IRP 486 to 9.0 ml of peptone diluent in a 17 x 100-mm snap-top tube. (This 1:10 dilution of antitoxin containing 50 AU/ml is stable when stored at -70° ± 5°C.)

3.3.3 Preparation of *C. perfringens* type C beta toxin

1. Prepare a 1:10 dilution of *C. perfringens* type C beta toxin by adding 1.0 ml of IRP 513(04) to 9.0 ml of peptone diluent. Dispense diluted toxin in 1.5-ml amounts into 13 x 100-mm tubes. IRP 513(04), diluted 1:10, is stable when stored at -70° ± 5°C.

4. Performance of the test

4.1 Vaccination of rabbits

4.1.1 Thoroughly shake each bottle of product and wipe the top with alcohol before filling the syringe.

4.1.2 Vaccinate each rabbit subcutaneously in the shoulder region with half of the largest recommended dose for any species indicated on the product label. Use 10-, 20-, or 30-cc syringes fitted with 20-gauge x 1-inch needles to vaccinate the rabbits.

4.1.3 For products that require 2 vaccinations, give the second vaccination 20-23 days after the first.

4.2 Collection and preparation of rabbit serum

4.2.1 Collect blood from the test rabbits 34-40 days after vaccination (or 14-17 days after the second vaccination for products that require 2 vaccinations).

4.2.2 Anesthetize rabbits for bleeding with a mixture of 1.32 mg/kg of xylazine and 8.8 mg/kg of ketamine hydrochloride. Give the anesthetic mixture by intramuscular injection.

4.2.3 Use a 12.5-ml serum separation tube fitted with a 20-gauge x 1 1/2-inch Vacutainer® needle to collect blood from the heart. Collect approximately 12.5 ml of blood from each rabbit. Gently invert tubes 5 times. Let the tubes of blood sit at 22°-26°C (room temperature) for 30-60 minutes.

4.2.4 Centrifuge blood at 1000 x *g* for 10-20 minutes at 22°-26°C (room temperature).

4.3 Preparation of serum pools

4.3.1 Prepare a pooled sample using an equal volume of serum from at least 7 rabbits per vaccinated group (provided that, if more than 7 rabbits are bled per vaccinated group, then equal volumes from each rabbit are used for the serum pool). If less than 7 rabbits are bled, the test is invalid and should be repeated.

4.3.2. The pooled sample may be held at 2°-7°C for up to 7 days. If testing will not be completed within 7 days, store the pooled sample at -20°C or lower.

4.3.3 Use 1.0 ml pooled serum to test for 10 AU/ml of antitoxin.

4.3.4 Dilute 1.0 ml pooled serum with 0.2 ml peptone diluent to test for 12 AU/ml of antitoxin.

4.4 Toxin neutralization

4.4.1 Preparation of *C. perfringens* type C standard beta toxin

Further dilute the *C. perfringens* type C beta toxin to 1:120 by adding 1.0 ml of diluted (1:10) toxin (see **Section 3.3.3.1**) to 11.0 ml of peptone diluent in a 17x120-mm conical tube. For the purpose of this test, the 1:120 dilution of IRP 513(04) is referred to as the standard beta toxin.

Note: A volume of 0.5 ml of standard beta toxin and 0.5 ml of peptone diluent represents 10 L_o doses. A volume of 0.8 ml of the standard beta toxin and 0.2 ml of peptone diluent represents 10 L_+ doses. For the purposes of this SAM, 10 L_o dose is defined as the greatest amount of toxin that, when mixed with 10 AU results in 100% survival of all mice inoculated intravenously (IV) with 0.2 ml of this mixture. The 10 L_+ dose is defined as the least amount of toxin that when mixed with 10 AU results in the death of 80%-100% of all mice inoculated IV with 0.2 ml of this mixture.

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4.4.2 Preparation of standard beta antitoxin

Thaw the *C. perfringens* type C beta antitoxin IRP 486, previously diluted 1:10 (see **Section 3.3.2**). Further dilute the toxin to 1:50 by adding 1.0 ml of diluted toxin (1:10) to 4.0 ml of diluent using a 17 x 100-mm snap-top tube. This dilution contains 10 AU/ml and is referred to as the standard beta antitoxin.

4.4.3 Product and standard beta toxin

1. Mix a sufficient volume of standard beta toxin and peptone diluent (0.5 ml of standard beta toxin and 0.5 ml of peptone diluent [10 L_o doses] for each serum pool and the L_o control) using a 17 x 120-mm conical tube. Add 1 ml of each serum dilutions to 1 ml of this standard beta toxin-peptone diluent mixture in 17 x 100-mm snap-top tubes. Mix each tube well using a vortex-type mixer.

2. Let the mixtures stand at 22°-26°C for 1 hour.

3. Place tubes in ice.

4.4.4 Standard beta toxin and standard beta antitoxin controls

1. Add 1.0 ml of standard beta antitoxin containing 10 AU/ml to 1.0 ml of standard beta toxin-peptone diluent mixture (10 L_o doses) in a 17 x 100-mm snap-top tube. Mix well with a vortex-type mixer.

2. Add 1.0 ml of standard beta antitoxin containing 10 AU/ml to a 17 x 100-mm snap top tube containing 0.8 ml of standard beta toxin and 0.2 ml of peptone diluent (10 L_+ doses). Mix well with a vortex-type mixer.

3. Let the mixtures stand at 22-26°C for 1 hour.

4. Place tubes in ice.

4.5 Inoculation of mice

4.5.1 Inject 0.2 ml of each standard beta toxin-product serum mixture into each of 5 mice.

4.5.2 Inject 0.2 ml of each standard beta toxin-standard beta antitoxin mixture into each of 5 mice.

4.5.3 Inoculate all mice into a lateral tail vein. Use 1-cc syringes fitted with 25- to 27-gauge x 1- to 1 1/4-inch needles.

4.5.4 Always inoculate the mice receiving the standard beta toxin-standard beta antitoxin mixtures (controls) **last**.

4.5.5 Mouse inoculations should be completed within 1 hour of placing the toxin-antitoxin mixtures in the ice.

4.5.6 The test is concluded 24 hours after the mice are inoculated.

5. Interpretation of test results

5.1 Criteria for a valid test

5.1.1 All 5 mice inoculated with the standard 10 L_o/10 AU control mixture must survive.

5.1.2 At least 4 of the 5 of the mice inoculated with the standard 10 L_+/10 AU control mixture must die.

Note: Moribund animals exhibiting clinical signs consistent with the expected disease pathogenesis that are unable to rise or move under their own power may be humanely euthanized and considered as deaths as outlined in 9 CFR 117.4.

5.2 Interpretation of test results

5.2.1 If 5 of the 5 mice inoculated with the undiluted pooled serum-standard toxin mixture survive, the serum contains at least 10 AU/ml of *C. perfringens* beta antitoxin, and the product is satisfactory.

5.2.2 If 5 of the 5 mice inoculated with the diluted (1.0 ml serum + 0.2 ml diluent) pooled serum-standard toxin mixture survive, the serum contains at least 12 AU/ml of *C. perfringens* beta antitoxin, and the product is satisfactory.

5.2.3 The product is considered unsatisfactory if the serum pool from at least 7 rabbits contains less than 10 beta AU/ml. (If any mice inoculated with the undiluted pooled serum and 10 L_o doses of standard beta toxin die, the product is considered to contain less than 10 AU/ml.)

6. Report of test results

Report results of the test(s) as described by standard Section operating procedures.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.111, U.S. Government Printing Office, Washington D.C., 2005.

7.2 History of toxin: *C. perfringens* type C culture #4414, used to produce IRP 513(04), and was obtained from Coopers Animal Health, Inc., 1201 Douglas Avenue, Kansas City, Kansas, on July 28, 1975. The number of passages is unknown.

7.3 History of antitoxin*: C. perfringens* type C (beta) antitoxin IRP 486 was produced in goats hyperimmunized with multiple injections of purified *C. perfringens* type C toxoid and toxin during a 6 month period.

8. Summary of revisions

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- IRP 448 has changed to IRP 486 throughout the document.
- IRP 447 has changed to IRP 513(04) throughout the document.
- Humane endpoint language has been added.
- Dilution/holding vessel sizes have been added for clarification.
- The contact person has been changed to Janet M. Wilson.